



Rapid Screening Analysis of Antioxidant Activities in Green Tea Products Using DPPH and FRAP

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ABSTRACT

Introduction: Free radicals are formed inside and outside the body. Free radicals inside the body can be created in the cell compartments. The reactions are complex through the initiation, propagation, and termination stages. On the other hand, free radicals in the outer body can be formed from pollutions and environmental damage action. Excessive free radicals cause oxidative stress and use destructive cellular to affect health conditions. Antioxidants are known as essential agents in biological systems against diseases triggered by free radicals. One of the many antioxidants found in tea plants (*Camellia sinensis*) is polyphenols.

Objectives: to describe the rapid screening analysis of the antioxidant activity methods using DPPH and FRAP in green tea drink.

Methods: A colorimetry method was employed to evaluate the dominant antioxidant compound of the total phenolic and flavonoid content in two selected tea products. The total phenolic content was determined by utilizing the Folin-Ciocalteu reagent. While an aluminum colorimetric assay is applied to assess flavonoid content. Rapid screening of antioxidant activity using a spectrophotometric method is employed to determine IC₅₀ values of tea extracts utilizing DPPH and FRAP.

Results: Their IC₅₀ score are 76,35 to 213,20 ppm in loose leaves tea and 87,92 to 269,79 ppm in the teabag (DPPH method); and 9,88 until 26,95 ppm in loose leaves tea and 10,47 to 19,57 ppm in the teabag (FRAP method).

Conclusions: The high total phenolic and flavonoid contents found in this experiment are in line with low IC₅₀ values or in other words high antioxidant activities.

Keywords: Antioxidant, *Camellia sinensis*, DPPH, FRAP

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1. Introduction

Free radicals are atomic particles that have one or more unpaired electrons. A side effect of excessive free radicals causes oxidative changes. When excess free radicals are in the body, they will play a role in cell damage through the oxidation metabolism. It can cause shutting down respiration cells through oxidizing membrane lipid, protein, and DNA pathways (1). Free radicals as reactive oxygen species (ROS) can occur inside (endogen) or outside the body (exogen). Endogenous radicals (e.g., peroxy ($\text{ROO}\cdot$), hydroxyl ($\text{OH}\cdot$)) are harmful and play a role in health and disease. Endogenous radicals are produced in cellular-compartments such as peroxisomes, cytosol, mitochondria, endoplasmic reticulum, plasma membrane, and lysosomes (2). The process is a complex series of chemical reactions. More details have been explained by Nandini Ghosh (2017). The formation of excessive exogenous radicals is the result of the involvement of human actions. Exogenous free radicals come from human actions that damage the environment and cause unhealthy lifestyles. They come from improper handling of industrial waste, increased motor vehicle fumes, land clearing by burning forests, excessive use of pesticides, and cigarettes smoke. Free radicals in foodstuffs can cause food deterioration. Chemical spoilage in food is marked by rancidity, change of color, flavor, and texture. It can cause deterioration of nutritional value and spoiled food (3). The reaction is spontaneous and is called autoxidation (1).

Open mindfulness of the threats of free radicals causes individuals to discover ways to neutralize free radicals through antioxidants. The Antioxidant is a helpful, economical, and convenient process. Antioxidants can donate electrons or hydrogen atoms to act as reducing agents. They are compounds that are capable of delaying, slowing, or inhibiting oxidation reactions in food and drugs whereas these compounds are easily oxidized so that other cells are protected from free radicals.

Base on their mechanism there are primary and secondary antioxidants. Primary antioxidants are also called chain-breaking antioxidants or scavenger antioxidants. Their action neutralizes radicals by donating electrons so that the radical compounds that have become stable cannot continue the propagation of the reaction, and the reaction chain is broken. Primary antioxidants are effective in the induction step and slow down the oxidation reaction. Examples of primary antioxidants are ascorbic acid, tocopherol, phenolic compounds such as gallic acid, and catechin. Secondary antioxidants prevent oxidation reactions by several mechanisms. The first mechanism inhibits initiation by oxidizing themselves. Secondary antioxidants can convert intermediate or final products into non-toxic products. For example, they prevent transition metal ions from participating in the reactions they catalyze.

Another secondary antioxidant's mechanism, such as ascorbic acid, encourages the rejuvenation of primary antioxidants by replenishing atom hydrogen (1).

Based on the source, antioxidants can be synthesized by chemicals or naturally found in animals, plants, and microorganisms. Some synthetic antioxidants have been made, such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene). It is commonly used as a preservative in food manufacturing. Plants as herbs, spices, fruits, vegetables, oil, and tea groups, provide natural antioxidants such as tocopherols, and polyphenols compounds. Polyphenol compounds act as antioxidants and are one of the usual sources of polyphenols in tea (*Camellia sinensis*). Tea is one of the foremost well-known drinks in the world. Tea is divided into green tea, black tea, and oolong tea (4). Green tea contains higher polyphenol content than the others. Many industries produce dried tea leaves for brewing and dried tea leaves that are packed in tea bags. Teabag is a small, porous, heat-resistant, and rope-filled pouch bag. It usually contains small fractions of tea leaves, while the loose leaves tea is dried as whole leaves and packed in plastic wrap or canned.

Antioxidant activity is determined in many strategic assays. The first type of method was developed to direct the measurement of hydrogen and electron transfer. They are related to their scavenging capacity against specific radical species. They are known as the free radical scavenging method (1). one of the specific radical species which is the most frequently used is 2,2-diphenyl-1-picrylhydrazyl (DPPH). It is based on the ability of phenol compounds to donate hydrogen atoms to DPPH free radicals. DPPH is the most common method to determine antioxidant capacity because it is fast, simple, accurate, low-costed, and sensitive (5,6,7). DPPH is a stable chromogen-free radical whose purple color changes when the reaction occurs. It is measured at 522 nm on a visible spectrophotometer. On the other hand, antioxidant activity is also able to be measured by a reduction-oxidation reaction. The capability of antioxidants as reductants was applied to reduce various metal ions. Iron, copper and chromium are the most commonly used. The Ferric Reducing Antioxidant Power (FRAP) method is based on the ability of antioxidants in reducing ferri-tripyridyl-triazine (Fe (III) TPTZ) to complex Ferro-Tripyridyl-Triazine (Fe (II) TPTZ) (8). The aim of this study is to describe the rapid screening analysis of the antioxidant activity methods using DPPH and FRAP in green tea drink.

2. Methods

Sample

The samples consist of six brands of tea products, marketed in Indonesia. They are GM, WA, CB, GA, TT, and DT. The abbreviations showed the origin

manufacturing of the green tea products which is from Ciwidey (Bandung), Pandaan (East Jawa), Pekalongan, Tegal, and Slawi (Central Jawa), respectively. They are all from Indonesia. The samples of this study are purchased at three supermarkets which are located in Tenggilis Mejoyo district, Surabaya. Each brand has two different types of tea products: green tea bags and loose leaves green tea.

Chemical Reagents

Ethanol 96% (Technical Grade), Filter paper (Whatman # 41, Maidstone England), demineralized water (University of Surabaya), Sodium hydroxide p.a., Gallic acid 98% (MP Biomedicals, France), 2,2-diphenyl-1-picrylhydrazyl (DPPH) p.a., 2,4,6-tripyrindyl-s-triazine (TPTZ), Catechin Standart p.a. (Sigma Aldrich, St. Louis, Missouri, US). Aluminium chloride p.a., Folin- Ciocalteu reagent, Sodium carbonate p.a., Anhydrous Sodium acetate, Acetic acid, and Ferric chloride (Merck KGaA, Darmstadt, Germany).

Equipment

The equipment used was spectrophotometer UV-Vis (Hitachi U-2000, Tokyo, Japan), analytical balance (Sartorius, Japan), micropipettes (Socorex, Swiss), glasswares (cuvettes, beaker glasses, volumetric flask).

Green Tea Extract

Green tea from the teabag was taken out from the teabag and weighed 250 mg. accurately, in a 50 ml beaker glass. On the other hand, 250 mg of loose leaves green tea was also weighed accurately in a 50 ml beaker glass. To each beaker glass, 25 ml of boiling demineralized water was added and allowed to stand for 10 minutes. Each green tea extract was filtered using Whatman no. 41 filter paper to a 50,0 ml volumetric flask. To the residue was added 20 ml boiling demineralized water and allowed to stand for another 10 mins. Then it was again filtered to the volumetric flask, and demineralized water was added to the mark (50,0 ml).

The number of tea leaves in this study is more dilute than that of the daily use of green tea drink. It should be noted that it is varied considerably in daily use of green tea among different ethnic populations in the world. 2-3 g of tea in Japan and China are brewed in 100-150 mL hot water. American people drink 2.25 g of tea in 180–240 mL water (9).

Total Phenolic Content

0,1 ml green-tea extract was pipetted into a 10,0 ml volumetric flask. Then 0,1 ml of Folin- Ciocalteu reagent was added into the volumetric flask and allowed to stand in the dark for 5 minutes. Furthermore, Na₂CO₃ solution (7%, 2 ml) was added to the volumetric flask and allowed

to stand in the dark for 30 minutes. After that, demineralized water was added to the volumetric flask up to a volume of 10,0 ml. The resulting blue solution was observed at the maximum wavelength (702,2nm). Three replications were performed. The result was expressed as Gallic Acid Equivalent (GAE) mg per 100 mg sample (10,11).

Total Flavonoid Content

1,0 ml green tea extract was pipetted into a 10,0 ml volumetric flask. Then 0,3 ml of 5% NaNO₂ solution was added and allowed to stand for 6 minutes. After that, 0,3 ml of 10% AlCl₃ solution was added and allowed to stand for another 6 minutes. Furthermore, 2,0 ml of 1M NaOH solution was added to the flask, and then demineralized water was added up to the mark. The absorbance of the pink solution was observed at the maximum wavelength. (507,5 nm). Three replications were performed. The result was expressed as Catechin Equivalent (CE) mg per 100 mg sample (8,12).

Measurement of Antioxidant Activity by DPPH Radical Scavenging Activity

1,0 ml green tea extract was mixed with 3,0 ml DPPH solution and 1,0 ml 80% ethanol in a vial, and it was kept in the dark (incubating). The sample solution was observed for its absorbance at the maximum wavelength of 522,0nm. A mixture of 3,0 mL of DPPH solution and 2,0 ml of 80% ethanol was used as control. Three replications have been performed. The following equation calculates the percentage of inhibition:

$$\% \text{ inhibition} = [1 - (\text{absorbance of test solution} - \text{blank absorbance}) / (\text{control absorbance})] \times 100\%$$

The % inhibition value of the various concentrations of the test solutions, a regression curve was made between the concentrations vs. % inhibition. Then the regression equation for the curve is determined: $y = bx + a$, where y = % inhibition, x = concentration of the test-solution, a = intercept, b = gradient. The IC₅₀ value can be calculated quantitatively by entering 50% as y value in the regression equation

Measurement of Antioxidant Activity with FRAP Assay

FRAP solution was prepared by dissolving 186.94 mg of anhydrous Na acetate in 1,6 ml of acetic acid (CH₃COOH) solution, and mineral-free water was added to make 100.0 ml buffer acetate solution (pH 3.6). On the other hand, 0,15 g TPTZ is accurately weighed and dissolved in 40 mM HCl until it reaches a volume of 50.0 ml (10 mmol/L TPTZ solution). Then 0,27 g of FeCl₃ 6H₂O was accurately weighed and dissolved in mineral-free water until it reached a volume of 50.0 ml (20 mmol/L FeCl₃ 6H₂O solutions). The final step was mixing and

homogenizing 25.0 ml of acetate buffer, 2,5 ml of TPTZ solution, and 2,5 ml of FeCl₃ 6H₂O solution (10:1:1) to produce FRAP reagent. 1,0 ml FRAP reagent was mixed with 1,0 ml green tea extract. The mixture was then put into a vial and allowed to stand according to the obtained stable time. Next, the absorbance was measured at the maximum wavelength (601,5 nm). Three replications were performed. The percentage of antioxidant capacity is calculated as the following equation:

$$\% \text{ capacity} = [1 - (10 - \text{Abs})] \times 100\%$$

The percentage value of the capacity of various concentrations of the test extract, a regression curve was made between the content vs. % capacity. Then the regression equation for the curve is determined $y = bx + a$, where: $y = \% \text{ capacity}$, $x = \text{concentration of test solution}$, $a = \text{intercept}$, $b = \text{gradient}$. The IC₅₀ value can be calculated quantitatively by entering 50% as y in the regression equation (13).

3. Result and Discussion

People in the world have recognized green tea as beverages. People believed that Green tea consumption is able to increase health promotion. Moreover, tea ritual time worldwide has become a culture country such as Japan, China, Korea, and The United Kingdom. After harvesting the leaves, green tea is processed by inactivating the enzyme polyphenol oxidase. The enzyme is heat - labile. Thus, the steam process was used to prevent oxidation reaction in the leaves. This process produced green tea extract which is abundant in flavonoids as well as the polyphenols. Tea leaves as loose-leaves tea are rolled, dried, and packed. While in the teabag, the leaves have to undergo a reducing size before being packed (9,14).

Polar or organic solvents such as methanol, ethanol, and acetone were often added to extract phenolic compounds, but Green tea leaves were only extracted with hot water in this study. This process is like serving tea in a drink. This process is quite effective because many of the polyphenolic components present in the vacuole can be simply extracted after the cell wall and membrane rupture. (15). The concentration of the extract in this study was 250 mg/ 50,0 mL or 5000 µg/mL, it was about a quarter of Japan's and China's serving or half of American's serving. The extracts in those servings are too concentrated for direct use in total phenolic, flavonoid, and antioxidant activity assay. Therefore, a dilution process was needed to facilitate the analysis process (9).

Total flavonoid contents as catechin equivalent were shown in table 1. The total flavonoid contents of six brands products were 1,57 to 4,83 mg in loose leaves tea and 2,83 to 5,61 mg in teabag. The variation of the content was caused by many factors. It depends on the different age of the leaves when it was picked and how the leaves were

processed such as fermentation, heating, and drying process. It affected their composition, especially flavonoids (14). As one of the subclass polyphenols, flavonoids have variations in substitution patterns of the hydroxyl groups. The total phenolic contents were expressed as gallic acid equivalent. This assay was based on the ability of the phenolic groups to be oxidized. This reaction makes Folin-Ciocalteu (contains phosphotungstic-phosphomolybdic) reduce to molybdenum-tungsten. The total phenolic contents analyzed in this study were 3,70 to 10,14 mg in loose leaves tea and 7,86 mg to 11,0 mg in teabag as gallic acid equivalent.

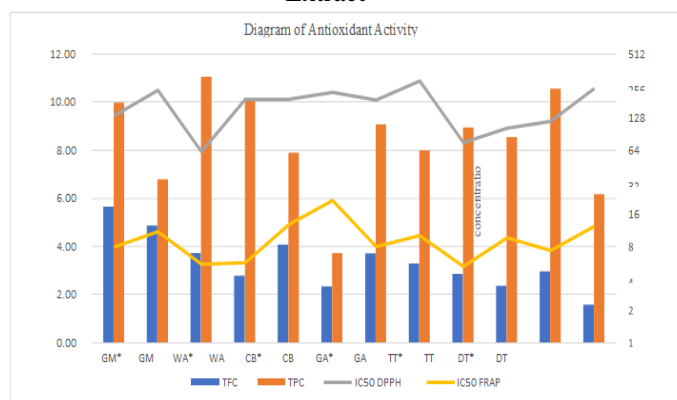
Regression curve of Gallic Acid : $y = 0,0913x + 0,0271$ $r^2 = 0,9962$ Regression curve of Catechin : $y = 0,018x + 0,006$ $r^2 = 0,9981$

Table 1. Total Phenol Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Activity of Green Tea

SAMPLE	TPC (% GAE)	TFC (%CE)	IC ₅₀ (ppm)	
			DPPH	FRAP
GM*	9,93±0,0729	5,61±0,012	150,85	28,96
GM	6,76±0,1003	4,83±0,056	76,35	11,33
WA*	11,00±0,1325	3,70±0,013	87,92	18,13
WA	10,14±0,0505	2,77±0,042	188,34	10,07
CB*	7,86±0,0747	4,04±0,008	370,69	19,57
CB	3,70±0,0302	2,31±0,007	213,2	26,32
GA*	9,03±0,0515	3,69±0,013	354,35	10,47
GA	7,96±0,1453	3,26±0,043	200,75	11,96
TT*	8,90±0,0421	2,83±0,048	241,45	26,95
TT	8,51±0,0337	2,34±0,014	105,65	9,88
DT*	10,51±0,1594	2,95±0,018	269,79	20,35
DT	6,14±0,1230	1,57±0,013	180,02	12,28

*Green tea bags

Figure 1. Total Phenol Content (TPC), Total Flavonoid Content (TFC), and Antioxidant Activity of Green Tea Extract



Antioxidant activity was represented in DPPH and FRAP methods as IC₅₀. IC₅₀ in the DPPH method can be defined as the effective concentration of the antioxidant compounds in tea that decreased initial DPPH concentration by 50% (1). It means that the DPPH method is used to determine radicals scavenging capacities. Antioxidant compounds in green tea donate their hydrogen atom (hydrogen atom transfer/ HAT) or transfer one electron to reduce radicals (single atom transfer/ SET). In the DPPH method, the combination of HAT and SET were performed for radicals scavenging determination (15). In the present study, antioxidants activities as IC₅₀ were shown in table 1 or graph 1. IC₅₀ in the DPPH method ranges from 76,35 to 213,20 ppm in loose leaves tea and 87,92 to 269,79 ppm in the green tea bag. Flavonoid groups in tea play a role in antioxidant activity. Hydroxyl substitution and flavonoid structure give various-level activity in antioxidants. The essential structure of flavonoids gives influential radicals scavenging activity such as the o-dihydroxy group in the B ring, C2-C3 double bond conjugated with a 4-keto group, and both 3-OH, 5-OH group in combination with a 4-carbonyl function (16).

On the other hand, IC₅₀ in the FRAP method means the effective concentration of the antioxidant compounds in tea that reduce Fe³⁺ to Fe²⁺ in FRAP by 50%. It only utilized the SET mechanism. Six products in loose leaves tea showed IC₅₀ from 9,88 until 26,95 ppm, and in the teabag was from 10,47 to 19,57 ppm. It was considered that other components in green tea could reduce metal ions in the redox mechanism, such as reducing sugar (17).

DPPH and FRAP could not be compared because they have different mechanisms to determine antioxidant activity. Their IC₅₀ has a similar pattern (graph 1). The high TPC and TFC values in loose leaves and tea bags (graph 1) largely is in accordance with low IC₅₀. Based on the important relationship between structure and effect of antioxidant activity, future studies are suggested.

4. Conclusions

Six products of green tea, which are marketed in Indonesia, have antioxidant activities which are from 76,35 to 213,20 ppm in loose leaves tea and from 87,92 to 269,79 ppm in the teabag (DPPH method) and from 9,88 to 26,95 ppm in loose leaves tea and from 10,47 to 19,57 ppm in the teabag (FRAP method). However, their antioxidant activities values using DPPH and FRAP have similar patterns. Flavonoid and polyphenol constituents have a role in antioxidant activity. It is shown that their high TFC and TPC values possess low IC₅₀. Or in other words, the lower IC₅₀ level, the higher the antioxidant activities.

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6. Conflict of Interest

There is no conflict of interest.

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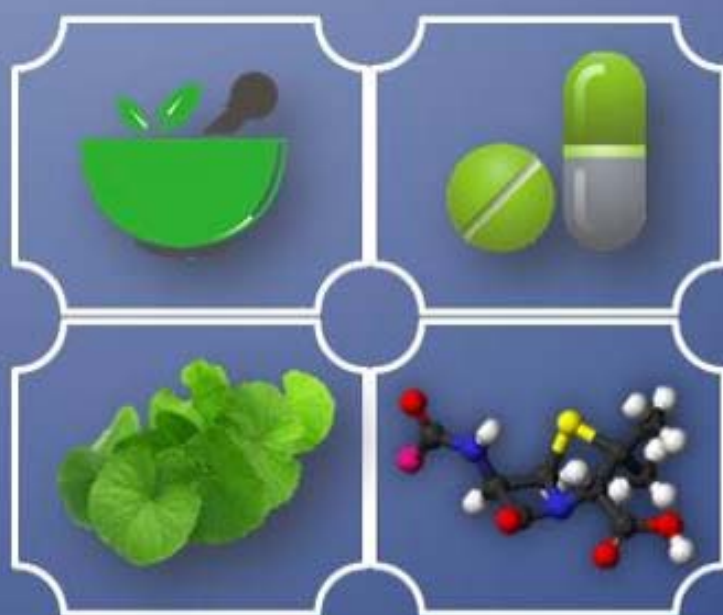
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